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By

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ABSTRACT

Leptin receptor (LepR) signaling in neurons of the ventromedial nucleus of the hypothalamus (VMH), specifically those expressing steroidogenic factor-1 (SF1), have been proposed to play a key role in controlling energy balance. By crossing LepR-silenced ($LepR^{loxTB}$) mice to those expressing SF1-Cre, we unsilenced native LepR specifically in the VMH and tested whether SF1 neurons in the VMH are critical mediators of leptin's effect on energy homeostasis. $LepR^{loxTB}$ x SF1-Cre (KO/Tg+) mice were metabolically phenotyped and compared to littermate controls that either expressed or were deficient in LepR. Leptin-induced pSTAT3 was present in the VMH of KO/Tg+ mice and absent in other hypothalamic nuclei. VMH leptin signaling did not ameliorate obesity resulting from LepR-deficiency in chow-fed mice. There was no change in food intake or energy expenditure when comparing complete LepR-null mice to KO/Tg+ mice, nor did KO/Tg+ show improved glucose tolerance. The presence of functional LepR in the VMH mildly enhanced sensitivity to the pancreatic hormone amylin. When maintained on high fat diet (HFD), there was no reduction in diet-induced obesity in KO/Tg+ mice, but KO/Tg+ mice had improved glucose tolerance after 7 weeks on HFD compared to LepR-null mice. We conclude that LepR signaling in the VMH alone is not sufficient to correct metabolic dysfunction observed in LepR-null mice.

INTRODUCTION

Leptin, a hormone mainly produced and secreted by the adipocytes of the white adipose tissue, acts in the brain to directly control body weight, food intake, and energy expenditure. Obesity in mice due to a deficiency of leptin (ob/ob) or its receptor (db/db) occurs together with hyperphagia, diabetes and aberrant metabolic homeostasis (20, 27, 33). Leptin exerts its actions by activating the b isoform of the leptin receptor (LepR), which is expressed in various brain regions involved in the control of energy homeostasis, including the arcuate nucleus (ARH) and the ventromedial nucleus of the hypothalamus (VMH) (9, 13, 16).

Leptin activates VMH neurons as demonstrated by their expression of phosphorylated STAT3 (pSTAT3), a marker of leptin signaling (9). Furthermore, many genes expressed in the VMH are involved in the control of energy homeostasis, notably steroidogenic factor-1 (SF1) (8, 21). SF1 is exclusively expressed in a subpopulation of VMH neurons, and is crucial for the correct development of the VMH and the formation of its efferent and afferent circuits (6, 7, 28). SF1 is also required for the expression of LepR and leptin-responsiveness in the VMH (8, 21). In rodents, deletion of LepR in SF1 neurons leads to heightened sensitivity to high fat diet, impaired glucose tolerance and increased levels of insulin and leptin (3, 9), suggesting that these neurons are protective against diet-induced obesity (DIO) and mediate leptin's glucoregulatory effects. The VMH is also the proposed brain site where leptin and the pancreatic hormone amylin interact to produce a synergistic reduction in body weight and food intake (19, 23). We recently observed reduced amylin sensitivity in LepR-deficeint db/db mice (11), leading to the hypothesis that reactivation of a single LepR population, such as VMH neurons, might restore amylin sensitivity.

While the requirement for native LepR in SF1 neurons in the VMH has been previously demonstrated (3, 9), their sufficiency has not. An earlier study, which overexpressed LepR in all SF1-positive cells of db/db mice, provided some indication of the sufficiency of LepR in the VMH—these mice weighed slightly less than db/db controls, but showed no improvements in food intake, body compostion or hyperglycemia (15). The observed decrease in body weight, with no corresponding change in food intake, led to the hypothesis that energy expenditure was increased. It is important to note, however, that this study overexpressed LepR in all SF1 neurons, even those that innately do no express LepR. To test exactly what contribution native VMH LepR neurons make to leptin's control of energy balance, we bred SF1-Cre mice to $LepR^{loxTB}$ mice, which have a loxP-flanked transcription blocking (TB) sequence inserted in the LepR gene (2), to produce mice with functional LepR exclusively in the SF1 neurons of the VMH. We evaluated the role of leptin signaling specifically in this VMH population in the control of body weight and composition, food intake, energy expenditure, glucose homeostasis, and amylin sensitivity. Because loss-of-function studies also demonstrated that SF1 neurons expressing LepR might mediate sensitivity to high fat diet (3, 9), we tested if leptin acting on native VMH LepR protects against DIO in mice challenged with a high fat diet.

MATERIAL AND METHODS

Generation of mice with unsilenced leptin receptors in SF1 neurons

To generate mice expressing native LepR exclusively in SF1 cells, LepR Cre-reactivatable mice ($LepR^{loxTB}$; see (2)) were crossed to mice expressing SF1-Cre. $LepR^{loxTB}$ breeders were purchased from Jackson Laboratory (Leprtm1Jke; Stock# 018989), and SF1-$Cre^{Tg/+}$ mice (9) were provided by Dr. Joel Elmquist of the University of Texas Southwestern Medical Center, and maintained by breeding transgenic male mice with C57BL/6J female mice.

Because homozygous $LepR^{loxTB/loxTB}$ mice are reportedly infertile, a three-level breeding strategy was designed in which the final cross of $LepR^{loxTB/WT}$ to $LepR^{loxTB/WT}$ x SF1-$Cre^{Tg/+}$ generated pups representing all four experimental conditions within single litters. The four experimental groups generated were: $LepR^{WT/WT}$ x SF1-Cre^{WT} (WT/WT), expressing normal LepR and no SF1-Cre; $LepR^{WT/WT}$ x SF1-$Cre^{Tg/+}$ (WT/Tg+), expressing normal LepR and SF1-Cre; $LepR^{loxTB/loxTB}$ x SF1-Cre^{WT} (KO/WT), LepR-null, equivalent to db/db mice, and expressing no SF1-Cre; and $LepR^{loxTB/loxTB}$ x SF1-$Cre^{Tg/+}$ (KO/Tg+), LepR-null, except for the reactivation of native LepR only in SF1-expressing cells of the VMH.

Mice were genotyped at the age of 6 or 7 days by double transgenic PCR analysis of toe DNA, which was determined using the following primers (Microsynth AG), as previously described (1, 12) : TGG CTT TTA AGC TCT GCA GTC ($LepR^{loxTB}$ common), CTG AGC TGC AGC GCA GGG ACA T (SF1 Cre Tg-F), TGC GAA CCT CAT CAC TCG TTG CAT (SF1 Cre Tg-R), TAG GGC CAA ACC CAC ATT TA ($LepR^{loxTB}$ WT) and CCC AAG GCC ATA CAA GTG TT ($LepR^{loxTB}$ KO).

Housing and environment

Male and female mice were housed in a temperature-controlled environment (21 ± 2°C) under a 12/12 hour light-dark cycle. Mice had *ad libitum* access to standard chow (Kliba Nafag 3430) and water, except when noted below. A subgroup of mice was maintained on high fat diet (HFD; 32% kcal from fat, D12266B from Research Diets) from approximately 5 to 14 weeks of age. The mice were group housed in macrolon cages until 5 to 6 weeks of age, when they were single housed in BioDAQ cages to continuously measure individual food intake. Meal pattern criteria were an inter-meal-interval (IMI) of 600 s and a minimum meal size of 0.02g. Each cage was furnished with a cardboard house including nest-building material. All experiments were performed with the approval of the Veterinary Office of the Canton Zurich, Switzerland, and in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

Feeding behavior tests

Between 8 and 12 weeks of age, male and female mice were fasted for 12 hours, and just prior to dark onset treated intraperitoneally (IP) with vehicle or amylin (20, 100, 1000 µg/kg in 0.9% NaCl; Bachem AG). There were four test days, each separated by at least 48 hours. On a given test day, each mouse was randomly assigned to a treatment group, excluding a treatment it had previously received. By the fourth test day, each mouse had received each treatment once. At dark onset, the gates of the food hoppers were reopened. Food intake was automatically measured and analyzed for 22-h post-injection.

Measurement of baseline energy expenditure

Following completion of feeding tests, mice were transferred to a 16-cage TSE PhenoMaster open circuit indirect calorimetry system for the determination of O_2 consumption and CO_2 production. Prior to each experiment, the system was calibrated using certified calibration gases, which allows the pooling and comparision of data from mulptiple experiments. Energy expenditure (EE) and respiratory exchange ratio (RER) were calculated based on equations from Weir (31). To account for differences in body weight and body mass composition, EE data were corrected for individual lean body mass (LBM in g) and fat mass (FM in g) using the following equation: LBM + 0.2FM, as recommended by Even and Nadkarni (14).

Intragastric glucose tolerance test (igGTT) and insulin measurement

At the ages of 8 and 12 weeks, basal blood glucose was measured and an igGTT was performed. Two hours before dark onset, food was removed, mice were lightly anesthetized under isoflurane, and blood was collected sublingually in a 100-ul EDTA-coated tube, prepared with a protease inhibitor (Sigma P2714), and processed for plasma. Plasma insulin and leptin was measured in duplicate using Mouse/Rat Metabolic assay according to manufacturer's protocol (N45ZA-1; Meso Scale Diagnostics). Baseline blood glucose was measured from whole blood (Glucometer: Breeze2, Bayer). Two hours later, a second baseline blood glucose was measured from tail blood. At dark onset, mice were gavaged with 50% glucose solution (2 g/kg), and blood glucose levels were measured from the tail 15, 30, 45, 60, 90 and 120 minutes later. In mice challenged with HFD, an igGTT was performed at 5 weeks of age, prior to access to HFD, and again at 12 weeks of age, after 7 weeks on HFD.

194 **Measurement of body composition**

195 CT scanning was performed using a La Theta LCT-100. The mice were
196 sutured closed after perfusion, and placed supine in the plexiglass holder.
197 The X-ray source tube voltage was set at 50kV with 1 mA current. A sagittal
198 image of the whole animal was acquired, followed by scans of the entire
199 animal. The region between the vertebrae L1-L6 was evaluated for lean and
200 fat mass.

201

202 **Perfusion for brain immunohistochemistry**

203 A second cohort of male and female mice was perfused at 8 or 20 weeks of
204 age to validate the expression of functional LepR in the VMH by assessing
205 leptin-induced pSTAT3. Mice were fasted for 2 hours, and at dark onset mice
206 were injected with leptin (3 mg/kg, IP; Peprotech). Mice maintained on HFD
207 were treated with leptin (5 mg/kg, IP) at 14 weeks old. Forty-five minutes
208 later, mice were deeply anesthetized with pentobarbital (200 mg/kg IP;
209 injection volume of 5 ml/kg), and mice were perfused transcardially by
210 hydrostatic forces with ice-cold potassium phosphate buffered saline (KPBS)
211 for 2 minutes to flush, and 2% PFA was used to fix the tissue. Brains were
212 removed and postfixed in 2% PFA for 1 h, and then cryoprotected (20%
213 sucrose solution in 0.1M PB) overnight at 4°C. Brains were then blocked into
214 hindbrain and forebrain, flash frozen in hexane on dry ice, and stored at -
215 20°C until sectioning.

216

217

218 **Immunohistochemistry for pSTAT3**

219 Frozen brains were sectioned through the VMH and ARH at 30 μm on a
220 cryostat (Leica CM3050S). Coronal brain sections were freeze-thaw mounted
221 on slides and stored in cryoprotective solution at -20°C until staining.

Brain sections were incubated with anti-pSTAT3 produced in rabbit (1:1000; #9145 from Cell Signaling Technology) for 48 hours at 4°C, and then incubated with Cy3-goat-anti-rabbit (1:200; Jackson Laboratories) for 2 hours at room temperature.

Quantification of pSTAT3

Sections were imaged at 20x magnification using Zeiss Axioimager Z2 epifluorescence microscope, fitted with Zeiss Axiocam HRm camera, and AxioVision Imaging System (Carl Zeiss MicroImaging GmbH, Germany). Images were batch processed using ImageJ and a customized macro. All images were captured using an exposure time of 270 ms together with linear image adjustment for the contrast and brightness. For each image, the region of interest (VMH or ARH) was manually selected, and the macro then transformed the image into a binary image by subtracting the background and thresholding the brightness. To separate coalesced cells, the watershed feature of ImageJ was used. The macro counted pSTAT3-positive cells using a minimum particle size of 30 pixels. To verify the quantification, an image overlay identifying each counted cell was generated and saved.

To assess leptin-induced pSTAT3 across the rostrocaudal extent of the VMH, we defined and quantified four anatomical levels of VMH and corresponding levels of ARH; Plate 67, bregma: -1.255 in Allen Mouse Brain Reference Atlas (10)) was defined as the first level, and the 3 subsequently caudal VMH sections, separated by steps of 120 µm (bregma levels: -1.375, -1.495, and -1.615), were levels 2 through 4. The average number of pSTAT3-postive cells across all levels was calculated and compared across groups. Though not quantified, other hypothalamic nuclei were assessed for the presence of

leptin-induced pSTAT3 to further verify the specificity of the SF1-targeted unsilencing.

Statistical analysis

Data are represented as mean ± SEM. Significance was tested using one-way or two-way ANOVA, followed by Tukey's multiple comparison, when appropriate (GraphPad Prism 7 for Mac). When normality was not achieved, nonparametric Kruskal-Wallis ANOVA was used for comparison. Because the comparison of KO/WT and KO/Tg+ was most relevant for the interpretation of our data, posthoc multiple comparisons were primarily made between mice of the same LepR genotype (i.e. WT/WT vs. WT/Tg+ and KO/WT vs. KO/Tg+). For the analysis of pSTAT3, igGTT, and amylin data, all groups were compared with posthoc testing. Total area under the curve (AUC) was calculated for igGTT data using the trapezoidal rule. A P-value < 0.05 was considered statistically significant.

RESULTS

Leptin sensitivity is restored in the VMH of $LepR^{loxTB}$ x SF1-Cre mice

Representative images showing the ARH and VMH of all groups at 8 weeks of age are shown in Figure 1A-D. At 8-weeks-old, we observed leptin-induced pSTAT3 in the ARH and VMH of chow-fed WT/WT and WT/Tg+ mice (Fig. 1E). In contrast, pSTAT3 immunoreactivity was completely absent in the ARH and VMH in KO/WT mice. KO/Tg+ showed a restoration of leptin-induced pSTAT3 in the VMH, but not the ARH. KO/Tg+ pSTAT3 levels were significantly higher than KO/WT in the VMH (Fig. 1E). Previous work showed that not all LepR-expressing neurons in VMH co-express SF1 (9), however, we found that KO/Tg+ mice had pSTAT3 levels in the VMH comparable to the

WT groups, with no significant differences between KO/Tg+ and LepR WT mice in the VMH. To determine if leptin signaling in the VMH diminishes with age, leptin-induced pSTAT3 was again assessed in 20-week-old chow-fed mice. At this time point, a pattern of pSTAT3 expression similar to the 8 week time point was observed in the ARH and VMH (Figs. 1F). In 14-week-old mice maintained on HFD for 9 weeks, leptin sensitivity was sustained in the ARH and VMH of WT/WT and WT/Tg+ mice, and in the VMH of KO/Tg+ mice (Fig. 1G). The numbers of pSTAT3-positive cells in the ARH and VMH were similar to chow-fed mice, however a higher dose of leptin was administered (5 mg/kg) to HFD-fed mice.

VMH leptin signaling does not ameliorate obesity or increased fat mass

On a normal chow diet (Figs. 2A & B), both male and female KO/WT and KO/Tg+ mice were significantly heavier than the WT mice from 6 weeks of age ($p<0.001$ for all comparisons), but were not significantly different from each other. When maintained on HFD for 6 weeks (Fig. 2C), we observed an early and faster body weight gain, which was comparable, in KO/WT and KO/Tg+ mice. From 6 weeks of age, LepR KO mice were significantly heavier than WT groups ($p<0.001$ for all comparisons), but never different from each other. These effects also did not vary with sex.

To determine whether restoration of LepR in SF1 neurons affected fat deposition, we performed CT scans postmortem in 14-week-old mice fed chow or HFD (Figs. 2D & E). Reactivation of LepR in SF1 neurons was insufficient to normalize body composition, regardless of maintenance diet. While lean mass was similar across all groups on both chow and HFD, fat mass was similarly increased in KO/WT and KO/Tg+ mice.

VMH leptin signaling does not normalize energy expenditure or food intake

We next assessed whether unsilencing of LepR in the VMH protects against reduced energy expenditure and hyperphagia observed in LepR-deficient mice. Hourly EE and the average EE for dark and light phases are shown in Figs. 3A & B. In the dark phase, there was a main effect of the LepR deletion, with both LepR KO groups expending significantly less energy than LepR WT groups on chow (F[1, 27] = 5.38, P < 0.05), but KO/WT and KO/Tg+ groups were not different from each other. No differences across groups were observed during the light phase. We observed a main effect of LepR on RER during both dark and light phases (F[1, 27] = 18.96 and 35.72, P < 0.001 for both; Figs. 3C & D); LepR KO groups exhibited consistently higher RER than LepR WT, but no differences between KO/WT and KO/Tg+ nor between WT/WT and WT/Tg+ were found. When maintained on HFD, the LepR-deficiency had a more pronounced lowering effect on EE, which was significant during both light and dark phases (F[1, 11] = 17.58 and 5.79, P < 0.01 and 0.05; Figs. 3E & F). However, the presence of VMH LepR had no effect on EE, compared to the KO/WT mice. As anticipated, all groups showed a reduction in RER when maintained on HFD rather than chow (Figs. 3G & H), with no detectable differences across groups in either phase of the light cycle.

Additionally, 24-h chow intake was similarly increased in KO/Tg+ and KO/WT, compared to LepR WT mice (main effect of LepR, F[1, 26] = 41.43, P < 0.001; Fig. 4A). The increased food intake resulted from an increase in meal size in KO groups (F[1, 26] = 15.60, P < 0.001; Fig. 4B), while there were no

329 differences in meal number across the four genotypes (F[1, 26] = 0.73, P >
330 0.05; Fig. 4C). When maintained on HFD, we observed similar differences in
331 food intake between the obese phenotypes and the control mice, but with no
332 differences between KO/WT and KO/Tg+ mice; 24-h intake of HFD was
333 higher in both LepR KO groups (main effect of LepR, F[1, 24] = 22.66, P <
334 0.001; Fig. 4D), as a function of increased meal size (F[1, 24] = 18.55, P <
335 0.001; Fig. 4E), with no change in meal number (F[1, 24] = 3.26, P > 0.05;
336 Fig. 4F).
337
338 **VMH leptin signaling mildly improves glucose tolerance in HFD-fed mice**
339 To investigate if VMH leptin signaling contributes to glucose handling, we
340 performed igGTT on 8-week and 12-week-old mice on chow diet (Figs. 5A &
341 B). There was no significant difference in fasting glucose (0 min time point)
342 across the four groups at either age. At 8 weeks, there was an overall effect
343 of genotype with an impaired glucose tolerance in the LepR KO mice (main
344 effect of LepR on AUC, F[1, 21] = 8.73, P < 0.01; Fig. 5A inset), however no
345 differences between KO/WT and KO/Tg+. At 12 weeks, we observed a
346 stronger overall effect of LepR genotype (main effect of LepR on AUC, F[1,
347 37] = 96.37, P < 0.001; Fig. 5B inset). Though AUC of KO/WT and KO/Tg+
348 were not different at this age, the glucose concentration at 45 min post-
349 gavage was significantly higher in KO/Tg+ than in the KO/WT group (P <
350 0.01), indicating that VMH leptin signaling worsened the rate of glucose
351 clearance after the challenge.
352
353 To further examine if VMH leptin signaling changes glucose tolerance under
354 conditions of DIO, the igGTT was again performed prior to and after 7 weeks
355 maintenance on HFD (Figs. 5C & D). Prior to HFD, we observed no
356 differences in baseline blood glucose, and a main effect of LepR genotype on

AUC (F[1, 24] = 35.39, P < 0.001; Fig. 5C inset), but no difference between KO/WT and KO/Tg+ mice. Following 7 weeks on HFD (Fig. 5D), however, KO/WT demonstrated significantly elevated baseline glucose compared to WT/WT and WT/Tg+ levels (P < 0.05 for both), but not KO/Tg+, and showed a delay in blood glucose levels returning to baseline, with levels significantly higher than KO/Tg+ mice at 60 min (P < 0.05). At the 90 (P < 0.001) and 120 min (P < 0.01) time points, only the KO/WT glucose levels were significantly higher than the mice expressing normal LepR. There was a main effect of LepR on AUC after HFD (F[1, 24] = 70.33, P < 0.001), but no differences between KO/WT and KO/Tg+ groups (P = 0.19). These data indicate, that despite having no effect on obesity or food intake, restoration of LepR in SF1 neurons leads to mildly improved glucose control when mice are maintained on HFD.

Baseline plasma insulin and leptin levels were measured in blood samples collected prior to the igGTT (Figure 5E and F). For all time points and diet conditions, both KO/WT and KO/Tg+ groups were hyperinsulinemic and hyperleptinemic compared to LepR-expressing control mice, and in most cases did not differ from each other. Following HFD consumption for 7 weeks, however, KO/Tg+ mice were significantly more hyperinsulinemic than KO/WT mice (P < 0.01). Leptin levels were above the level of detection for two KO/Tg+ mice and not included in the analysis.

VMH leptin signaling marginally improves amylin sensitivity

Based on evidence showing that LepR-deficient mice have reduced amylin sensitivity (11) and that the VMH may mediate leptin and amylin interaction in rodents (23), we tested whether restored VMH leptin signaling would enhance

384 the anorexic response of peripherally administered amylin. The dose-
385 dependent reduction in food intake 1, 2, 4, and 22 h after various doses of
386 amylin is shown in Figs. 6A-D. In order to directly compare the effectiveness
387 of amylin to reduce food intake in all genotypes, data were expressed as a
388 percent of baseline food intake (vehicle treatment = 100%; Figs. 6E-H). One,
389 2, and 4 hours after amylin treatment, we observed a significant main effect of
390 amylin (F[3, 162] = 16.07, 8.02, and 13.28, $P < 0.001$ for each); at 2 hours,
391 there was a main effect of genotype (F[3, 162] = 3.00, $P < 0.05$). Post-hoc
392 analysis showed that after 1 hour, 100 μg/kg of amylin significantly
393 suppressed food intake in the WT/WT, WT/Tg+ and KO/Tg+ mice ($P < 0.05$
394 for all; see Fig. 6E), but not the KO/WT group. The dose of 1000 μg/kg of
395 amylin suppressed food intake in all groups after 1 hour (WT/WT: $P < 0.001$;
396 WT/Tg+ and KO/Tg+: $P <0.01$; and KO/WT: $P < 0.05$), but only in the WT/Tg+
397 and KO/Tg+ groups after 2 hours ($P < 0.05$ for both; Fig. 6F). Four hours after
398 treatment (Fig. 6G), food intake was reduced in the WT/Tg+ after 20 μg/kg of
399 amylin ($P < 0.05$), in WT/WT and WT/Tg+ after 100 μg/kg of amylin ($P < 0.01$
400 and $P < 0.05$, respectively), and in all groups after 1000 μg/kg of amylin ($P <$
401 0.05 for WT/WT and KO/WT; $P < 0.01$ for WT/Tg+ and KO/Tg+).
402

DISCUSSION

The current study aimed to characterize the role of leptin signaling specifically on VMH neurons in controlling energy and glucose metabolism. To achieve this, innate LepR expressed only in SF1 neurons were unsilenced in otherwise LepR-deficient mice. While we could demonstrate that leptin-induced pSTAT3 was present only in the VMH and not other hypothalamic nuclei, restoration of LepR in SF1 neurons did not prevent the development of an obese phenotype. VMH leptin signaling did not improve the increased fat accumulation, increased food intake, reduced energy expenditure, nor impaired glucose homeostasis, which was observed in a completely LepR-null KO/WT mouse. Furthermore, while leptin action in the VMH is hypothesized to protect against diet-induced obesity (3), we observed few benefits of VMH LepR signaling when mice were challenged on HFD. As on chow, KO/Tg+ remained as obese as KO/WT mice, again demonstrating increased food intake and reduced energy expenditure. Interestingly, unsilencing VMH LepR appeared to improve glucose tolerance in mice maintained on HFD, suggesting that leptin signaling in SF1 neurons could be relevant for the control of glycemia under pathological conditions, such as obesity. Overall, our findings definitively demonstrate that leptin signaling in SF1 neurons of the VMH is not sufficient for the control of energy homeostasis.

Earlier reports, which eliminated leptin signaling from SF1 neurons, provided evidence that LepR expressed in SF1 neurons are necessary controllers of energy homeostasis. Genetic deletion of LepR from SF1 neurons produced KO mice that were heavier than WT controls, an effect that was more pronounced on HFD, and displayed disturbed glucose tolerance (3, 9). When challenged with HFD, KO mice showed a mild increase in food intake, but a

clear impairment in the thermogenic response to the HFD. Together, the published data suggested that deficiency in LepR in SF1 neurons leads to increased susceptibility to DIO, which results from impaired control of food intake and energy expenditure (3, 9).

Because of these findings, we hypothesized that re-activation of native LepR in SF1 neurons would promote metabolic health and protect against obesity, but this is not what we observed. A previous study investigated the result of overexpression of LepR in all SF1-expressing neurons, and observed a slightly leaner phenotype than db/db mice, with no correction of diabetes or elevated glucagon levels (15). Unlike our model, in which only natively-expressed LepR were unsilenced from SF1 neurons, this model produced an overexpression of LepR in all SF1 neurons, resulting in a heightened leptin-induced pSTAT3 in the VMH (15). Because leptin does not seem to uniformly activate or inhibit SF1 neurons (25), the net output of these overexpressed LepR and how it relates to the net output of the native population was not entirely clear. Results from the overexpression study, showing reduced obesity with no change in food intake, led to the hypothesis that LepR signaling in SF1 neurons increases energy expenditure (15). Our results, however, do not confirm this hypothesis.

While it is possible that LepR expressed on SF1 neurons truly are not critical mediators of metabolic state, we propose two potential explanations for our primarily null findings. First, leptin signaling in the VMH is complex, and it is possible that unsilencing innate LepR in the SF1 neurons may not restore all functions of these neurons or this nucleus in its entirety. It is known that up to 20% of leptin-activated neurons in the VMH are negative for SF1 (9), and because our model should not reactivate expression of LepR in non-SF1

neurons, we can only speculate how these discrete populations interact or influence downstream behavioral or physiological output. In a recent report, Meek and colleagues hypothesized that this non-SF1 LepR population may influence the net output of SF1/LepR neurons (22), an idea which is further complicated by the fact that the VMH contains SF1 neurons that are both depolarized and hyperpolarized by leptin (25). Future investigations into the microcircuitry of SF1 LepR neurons and the role of non-SF1 neurons expressing the LepR within in the VMH are necessary to understand why selective reactivation of native LepR on SF1 neurons in the VMH has little consequence on the obese phenotype of LepR-null mice.

A second interpretation is that this population of LepR cannot function in isolation, nor can it compensate for the severe metabolic disturbance produced by the absence of all other LepR populations. It is possible that leptin signaling via SF1 neurons requires additional populations of functional LepR, such as in the ARH or non-SF1 neurons in the VMH, in order to induce changes in metabolism. It is established that postnatal ARH leptin signaling is required for correct organization of hypothalamic circuitry (5). Leptin-deficient ob/ob mice and LepR-deficient db/db mice show disturbed neuronal projections from the ARH to the paraventricular nucleus of the hypothalamus, which is a critical pathway in the control of energy balance (4). This phenotype is presumably replicated in our KO/WT and KO/Tg+ mice, which lack ARH LepR. As the ARH is a known target of VMH projections (26), and a potentially critical mediator of the VMH's effects on energy balance, it is possible that disturbed ARH circuitry halts downstream transmission of the leptin signal generated in the VMH of KO/Tg+ mice. A more direct relationship between ARH and VMH LepR signaling might also exist; co-activation of these ARH POMC neurons by leptin could be necessary to transduce the

hypothesized anorexic action that follows SF1 leptin signaling. Interestingly, reactivation of LepR exclusively in POMC neurons also did not correct the hyperphagia observed in LepR-null mice, and only modestly corrected obesity (2), which would support the hypothesis that both LepR populations are required for leptin's full effect on food intake and body weight control.

KO/Tg+ mice showed a slight improvement in glucose tolerance compared to the KO/WT mice, but only when they were maintained on HFD. Because the KO/Tg+ and KO/WT mice on HFD did not differ in body weight or food intake, it is possible that restoration of LepR in the VMH directly influenced insulin signaling in the VMH to alter glucose metabolism. It was previously shown that LepR-deficient db/db mice have reduced insulin-induced pAkt in ARH neurons, corroborating that functional leptin signaling is important for insulin signal transduction (18). Interestingly, activity of insulin-responsive SF1 neurons was shown to also vary with diet (17). These neurons are overactive specifically under conditions of HFD-feeding and concomitant hyperinsulinemia, two conditions present in our KO/Tg+ mice, which results in elevated PI3K activation and a proposed inhibition of the glutamatergic VMH projections to ARH POMC neurons (17). While VMH insulin signaling should not theoretically be disrupted in any of our experimental groups, it is possible that the effect on glucose tolerance we observed in the KO/Tg+ mice results from an interaction between the HFD-induced hyperactivity in insulin-responsive SF1 neurons and enhanced pAkt signaling in these neurons, which could be a secondary result of unsilencing of VMH LepR.

Because obesity-induced leptin resistance is a major hurdle in developing leptin-based therapies to combat obesity, the pancreatic hormone amylin received significant attention for its capacity to restore leptin sensitivity in

obese humans and rats (23, 24, 29). It was proposed that amylin and leptin synergize by activating complementary, but discrete, neuronal signaling pathways, and that the VMH is a critical locus of convergence for these pathways (23, 30). While much of this research has focused on how amylin sensitizes leptin, our recent findings showed that LepR-deficient mice (db/db) and rats (ZDF) have reduced amylin-induced satiation, and that db/db mice also exhibit reduced amylin-induced Fos in the primary target nucleus of amylin, the area postrema (AP; 11), suggesting a bi-directional dependency of these hormones. We were therefore interested in knowing if restoring LepR function in a specific neuronal population could restore amylin sensitivity, and hypothesized that reactivation of LepR in SF1 neurons would enhance amylin signaling in LepR-deficient rodents. Similar to the db/db mice in the previous study (11), the KO/WT mice exhibited an altered dose-response to amylin; notably, the 100 μg/kg dose failed to reduce food intake in these mice. In contrast, by restoring VMH LepR signaling, the KO/Tg+ group exhibited a dose-response to amylin more similar to the LepR WT groups, with the 100 μg/kg dose of amylin reducing food intake. Whether this effect is physiologically significant will require further investigation. Interstingly, while only statistically significant in the LepR WT groups, all groups demonstrated reduced food intake at the 4-hour time point. Because amylin in the blood has a very short half life of less than 15 min (32), it is known that the “fast” or direct action of amylin is quite short, and its effect on food intake begins to dissipate 2 hours after administration. This revived reduction in food intake at 4 hours, most notable in the KO/WT group which showed little suppression prior to this time point, suggests the presence of a “slow” action of amylin, one which could be independent of leptin action.

PERSPECTIVES AND SIGNIFICANCE

We observed that LepR signaling specifically in SF1 neurons of the VMH is not sufficient to mediate the metabolic effects of leptin in animals that are otherwise LepR deficient. VMH LepR signaling, however, improved glucose homeostasis under HFD conditions. These findings suggest that SF1 neurons in the VMH might contribute to the regulation of leptin-mediated glucose homeostasis following diet-induced obesity. The presence of SF1 LepR also enhanced responsiveness to acute amylin treatment. Further studies should address whether leptin signaling in the VMH plays a subordinate role in metabolic regulation or if its functions depend on connections with other leptin-regulated nuclei in the brain.

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AUTHOR CONTRIBUTIONS

TAL and CNB designed the experiments; SSS, CLF, LW, ET, SD and CNB performed the research and reviewed the manuscript; SSS, TAL and CNB wrote the manuscript. TAL and CNB are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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686 **FIGURE LEGENDS**

687

688 **Figure 1. LepR are only unsilenced in the VMH of KO/Tg+ mice.**
689 Representative fluorescent photomicrographs of leptin-induced (3 mg/kg)
690 pSTAT3 immunoreactivity in 8-week-old WT/WT (A), WT/Tg+ (B), KO/WT
691 (C), KO/Tg+ (D) mice fed with chow. Mean ± SEM number of pSTAT3
692 positive cells across four anatomical levels of ARH and VMH in 8-week-old
693 (E) and 20-week-old (F) male and female chow-fed mice. N = 5-6 mice/group.
694 Mean ± SEM number of pSTAT3 positive cells across four anatomical levels
695 of ARH and VMH in 14-week-old male and female mice fed with HFD for 9
696 weeks (G). N = 4-7 mice/group. Scale bar = 150 μm. Fluorescent images
697 were inverted to improve visibility. Symbols denote significant differences
698 between KO/WT vs. KO/Tg+; *P<0.05, **P<0.01 as determined using
699 Kruskal-Wallis ANOVA followed by Dunn's multiple comparison test.

700

701 **Figure 2. Unsilencing of LepR in SF1 VMH neurons has no effect on**
702 **body weight or composition.** Mean ± SEM weekly body weight of male (A;
703 N = 8-10 mice/group), and female (B; N = 5-6 mice/group) mice on chow diet,
704 and male and female mice on high fat diet (C; N = 5-8 mice/group). Mean ±
705 SEM mass of lean, visceral fat, and subcutaneous fat as measured by CT
706 scan in 14-week-old male and female mice maintained on chow (D; N = 7-9
707 mice/group) or HFD (E; N = 5-8 mice/group). D and E also depict the
708 correction factor used to normalize energy expenditure data, which was
709 calculated using lean body mass (LBM) + 0.2 fat mass (FM). The region
710 between the vertebrae L1-L6 was evaluated for lean and fat mass.
711 Comparisons of body weight and composition were made using two-way
712 ANOVA followed by Tukey's multiple comparison.

713

714 **Figure 3. Selective reactivation of LepR in SF1 VMH neurons does not**
715 **improve energy expenditure (EE) or respiratory exchange ratio (RER).**
716 Mean ± SEM energy expenditure (A, B, E, F) and respiratory exchange ratio
717 (C, D, G, H) calculated hourly (A, C, E, G) and during dark and light phase (B,
718 D, F, H) measured in WT/WT (N = 9), WT/Tg+ (N = 7), KO/WT (N = 6), and
719 KO/Tg+ (N = 9) male and female mice fed standard chow (A-D) or WT/WT (N
720 = 4), WT/Tg+ (N = 3), KO/WT (N = 4), and KO/Tg+ (N = 4) male and female
721 mice fed high fat diet (E-H). Symbols denote significant main effects of the

LepR: *P<0.05, **P<0.01, ***P<0.001 as determined using two-way ANOVA followed by Tukey's multiple comparison.

Figure 4. Selective reactivation of LepR in SF1 VMH neurons does not reduce food intake or alter meal patterns. Mean ± SEM 24-hour food intake (A, D), meal size (B, E) and meal number (C, F) measured in WT/WT, WT/Tg+, KO/WT, and KO/Tg+ male and female mice fed standard chow (A-C; N = 5-10 mice/group) or high fat diet (D-F; N = 5-8 mice/group). Comparisons were made using two-way ANOVA followed by Tukey's multiple comparison.

Figure 5. VMH leptin signaling mildly improves glucose tolerance in HFD-fed mice. Mean ± SEM blood glucose concentrations measured during intragastric glucose tolerance test (igGTT) in WT/WT, WT/Tg+, KO/WT, and KO/Tg+ male and female mice fed chow diet at 8 weeks (A; N = 3-9 mice/group) and 12 weeks of age (B; N = 9-12 mice/group). igGTT was repeated in a second cohort of mice at 5 weeks of age (C; N = 5-8 mice/group), and age at 12 weeks (D; N = 5-8 mice/group) after 6 weeks on HFD. Insets show mean ± SEM AUC for glucose levels during the 120-min test. Prior to glucose gavage, plasma insulin (E) and leptin (F) were measured in male and female mice maintained on chow (N = 7-10 mice/group) or HFD for 6 weeks (N = 3-8 mice/group). Symbols denote significant differences between KO/WT and KO/Tg+: *P<0.05, **P<0.01, or significant differences between KO/WT and WT/WT and WT/Tg+: ††P<0.01, †††P<0.001 as determined using two-way ANOVA followed by Tukey's multiple comparison.

Figure 6. VMH leptin signaling marginally improves amylin sensitivity. Mean ± SEM cumulative food intake in WT/WT (N = 15), WT/Tg+ (N = 12), KO/WT (N = 10) and KO/Tg+ (N = 9) male and female mice at one (A), two (B), four (C) and twenty-two hour (D) after treatment with vehicle, 20, 100 or 1000 µg/kg amylin (IP). Food intake data represented as a percentage of vehicle baselines in WT/WT, WT/Tg+, KO/WT and KO/Tg+ are shown in E-H. Symbols denote significant differences from vehicle treatment: WT/WT *P<0.05, **P<0.01, ***P<0.001; WT/Tg+ †P<0.05, ††P<0.01; KO/WT

759 §P<0.05; KO/Tg+ ^P<0.05, ^^P<0.01 as determined using two-way ANOVA
760 followed by Tukey's multiple comparison.
761

Figure 1

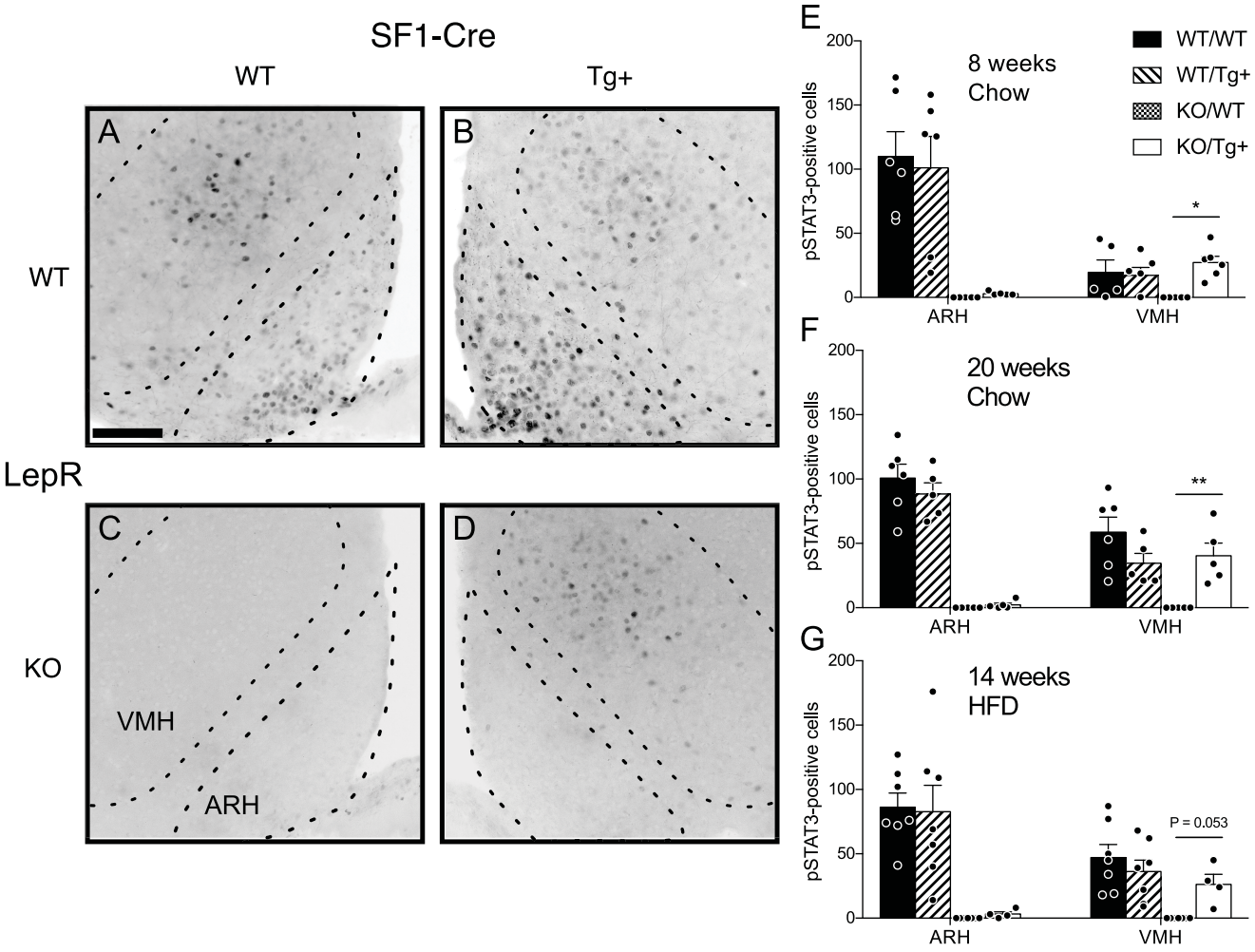
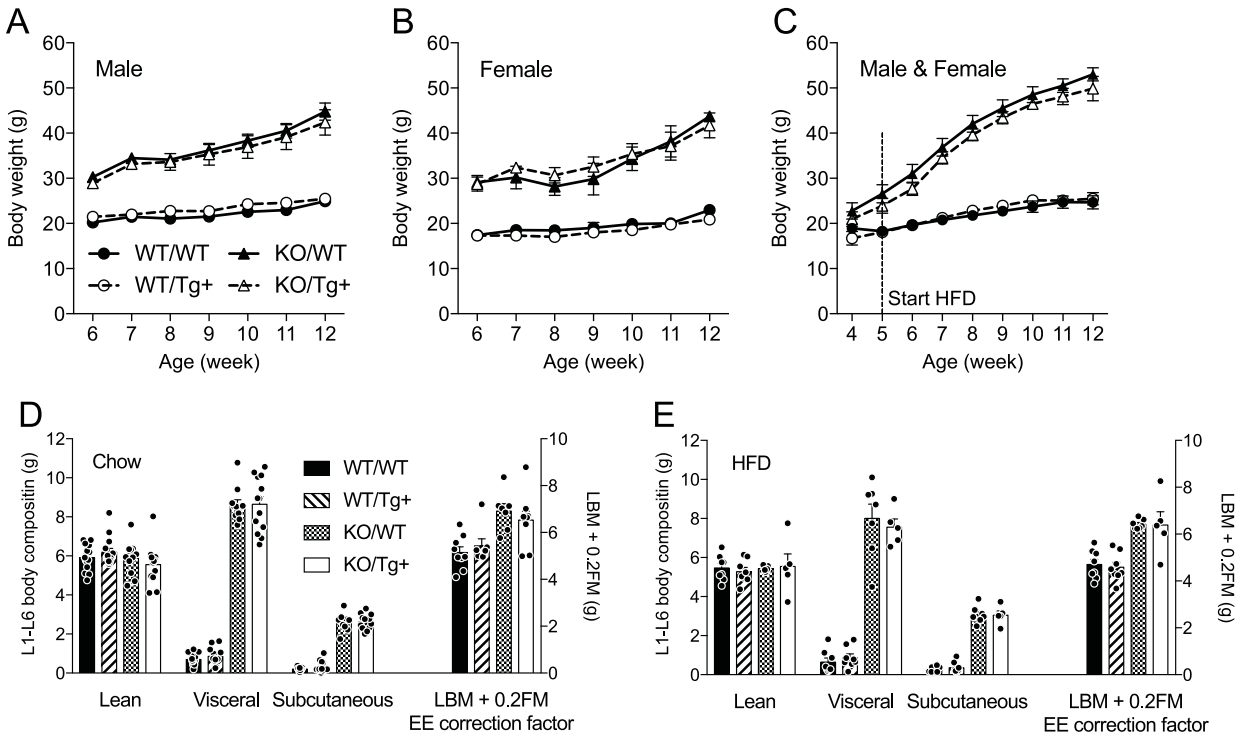


Figure 2



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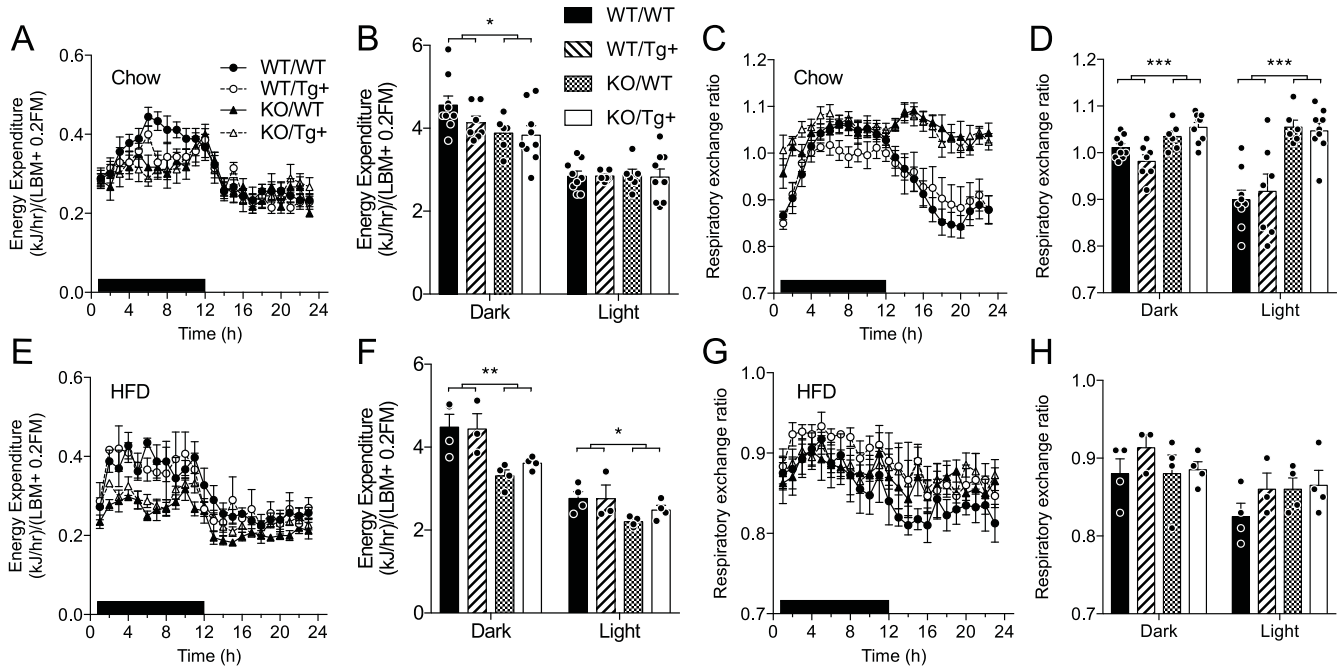

Figure 4

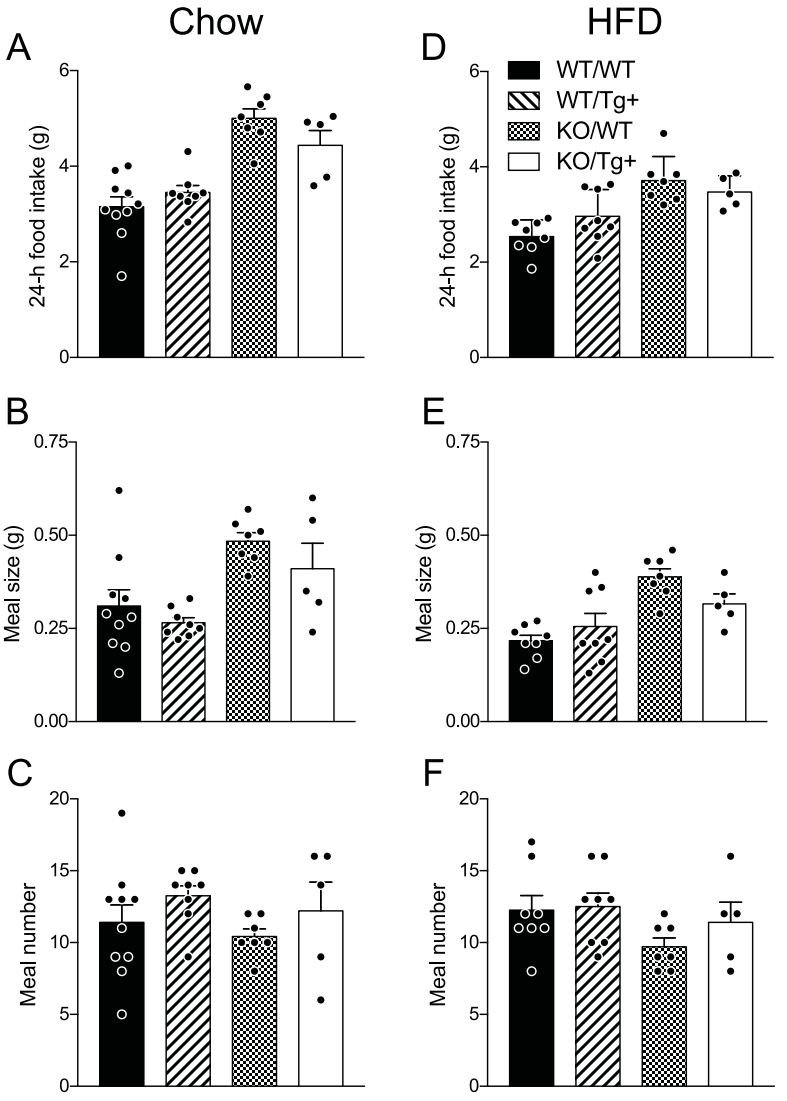

Figure 5

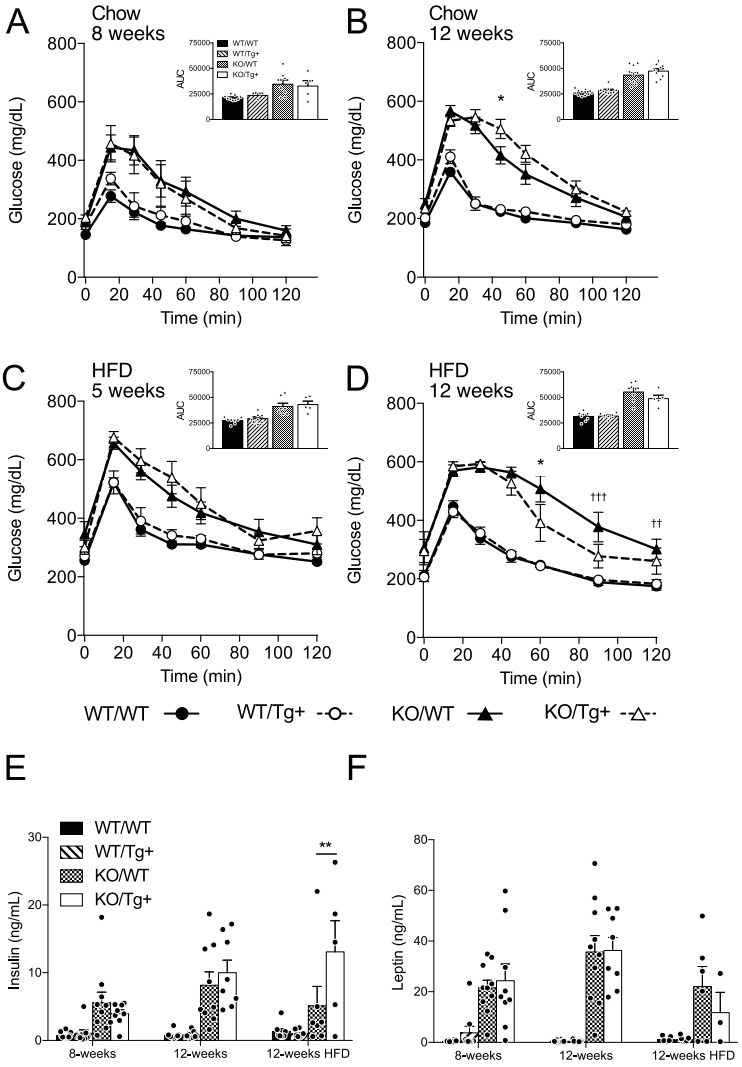

Figure 6